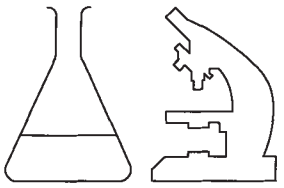
Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

PROPAGATION OF VIRUSES



Contents

Animal

Steffen Faisst, Novo Nordisk AIS, Soeborg, Denmark

Introduction

Viruses exist in two functionally distinct forms. The viral particle, referred to as virion, represents the static extracellular form without any metabolic activity, serving as a vehicle for the viral genetic material. The second, the dynamic form, consists of the viral genetic material itself once it is uncoated in a host cell. This active form of a virus uses the host cell biosynthesis machinery and energy supplies to multiply and to generate progeny virions. Hence, virus multiplication exploits host cells in a parasitic way. This results in the disturbance of normal cellular functions in many virus-infected cells, one of the basic mechanisms of viral diseases.

Virus propagation under defined laboratory conditions provides an important experimental tool in basic research in virology, allowing studies of virus multiplication, virus–host cell interactions and viral pathogenesis. Furthermore, virus propagation provides the basis for diagnoses of viral disease and vaccine production, as well as the generation of recombinant viruses for potential use in gene therapy.

Here, the mechanisms of intracellular virus multiplication are summarized, and techniques used for virus propagation, purification and titration are described.

The Virus Multiplication Cycle

To multiply, a virus has to enter a living cell. Thereafter, the viral genome is released from the capsid, and interacts with the host cell in order to replicate and to produce viral proteins. New capsids are assembled, and the newly synthesized genomes are packaged into these capsids either concomitant with or after their assembly. This results in progeny virions, which are released from the cell in order to transfer the viral genome to new host cells.

The initial step of virus–cell contact, referred to as adsorption, is mediated by the binding of a viral protein, located at the virion surface, to a receptor at the cell surface. Cellular receptors of many viruses have been identified. Most viruses adsorb to cell surface proteins that have specific metabolic functions, and that are expressed only in a subset of differentiated cells. Rabies virus, for example, binds to the acetylcholine receptor, and accordingly adsorbs to nerve cells. Human immunodeficiency virus adsorbs to CD4 molecules of T lymphocytes and macrophages, but needs co-receptors for viral entry into these cells. These co-receptors have been identified recently, and belong to the family of chemokine receptors. It should also be noted that carbohydrates have been shown to act as virus receptors. For example, polyomavirus, Sendai virus and vaccinia virus adsorb to sialyloligosaccharides of glycoproteins and glycolipids, which can be found on the surface of many cell types.

After adsorption, viruses have to cross the plasma membrane in order to enter a cell. The mechanism of penetration differs from virus to virus, depending on the respective virion structure. Adsorption to cell receptors brings viruses into intimate contact with the plasma membrane. Subsequently, viruses with a membranous envelope may directly inject the viral capsid into the cytoplasm by membrane fusion. However, the virus–receptor complex can also be internalized by coated pit-mediated endocytosis and delivered to endosomes. Subsequent fusion of viral and endosome membranes leads to the release of the viral capsid into the cytoplasm.

Viruses lacking an envelope also enter cells by coated pit-mediated endocytosis, but entry of the capsid into the cytoplasm obviously cannot occur via membrane fusion. For the non-enveloped adenovirus, evidence has been presented that capsid proteins induce the lysis of endosomes, thus releasing the capsid into the cytoplasm.

Once within the cytoplasm, the viral genome has to be liberated from the capsid and transported to the appropriate intracellular site for transcription or replication. This event is referred to as uncoating.

For RNA viruses, cellular factors like proteases are thought to dismantle the virus capsids immediately after entry, releasing the viral RNA into the cyto-

plasm, where it is replicated and translated. Also poxviruses, which contain a double-stranded DNA genome, replicate in the cytoplasm, using a virus-encoded DNA polymerase. Only retroviruses transcribe their RNA genome in DNA, which subsequently translocate to the nucleus and integrate into the cellular genome. The integrated retroviral genome serves as a template to synthesize new RNA genomes.

For nuclear-replicating DNA viruses, the capsid is supposed to be translocated along the cytoskeleton to the nuclear pores. At the nuclear pores, the viral DNA is released into the nucleus, where it is replicated.

When the viral genome is delivered to its appropriate intracellular site, it has to interact with the host cell biosynthesis machinery in order to get amplified and transcribed into mRNAs allowing the synthesis of viral (capsid and noncapsid) proteins. Thereafter, cellular and viral factors mediate the assembly of the capsids, and the packaging of the viral genome into these capsids. These steps are referred to as capsid maturation. The assembly of capsid proteins of nonenveloped viruses occurs either in the nucleus (e.g. parvoviruses, adenoviruses and papovaviruses) or in the cytoplasm (e.g. reoviruses). Most of the nonenveloped viruses rely on host cell lysis for their egress.

The assembly of the capsids of enveloped viruses also takes place either in the cytoplasm (most RNA viruses, poxviruses), or in the nucleus (herpesviruses), but the last step in virion assembly is linked to the release of the virions. Most RNA viruses egress by budding through the plasma membrane. Herpesviruses have been shown to leave the nucleus by budding through the inner nuclear membrane. They are then released from the cells by transport through the endoplasmic reticulum. Poxviruses may be released from the cells either by budding through the Golgi apparatus, or by cell disruption, resulting in enveloped and nonenveloped particles, respectively. Both enveloped and nonenveloped particles are infectious. Once progeny virus has been released, new cells can become infected, and further multiplication cycles are initiated.

Cytopathic Effect

Different types of interaction between viruses and host cells can be observed. Many viruses kill or morphologically modify their host cells when they multiply. This is called the cytopathic effect (CPE), and the respective virus is said to be cytopathogenic.

Generally, cytopathogenic viruses code for proteins that shut off synthesis of cellular macromolecules, or that are cytotoxic. Furthermore, the capsid proteins of nonenveloped viruses seem to be implicated in cell lysis, on which these viruses may rely for their egress. Enveloped viruses may additionally insert viral proteins into the cell membrane, which also may impair the viability of the target cells. Cell death within a few days is the result of productive infection with many types of viruses, such as togaviruses, picornaviruses or autonomous parvoviruses. Infection with poxviruses, reoviruses or adenoviruses also leads to cell death, but less rapidly.

Another type of CPE is the induction of cell fusion by viruses such as paramyxoviruses, human immunodeficiency viruses and herpesviruses. Induction of cell fusion is also due to the insertion of viral proteins into the host cell membrane, and results in the formation of syncytia (giant cells with up to several hundred nuclei).

As a result of mild CPEs, a balance between cell growth and virus production can sometimes be observed (e.g. after paramyxovirus infections). Such 'carrier cultures' may be seen as the cell culture counterparts of chronic infections in animals. Several RNA viruses that are not cytopathogenic (like arenaviruses and most retroviruses) may also provoke such steady-state infections.

Host Range, Permissiveness and Susceptibility

Any cell that can be infected by a virus is said to be susceptible. However, infection of a susceptible cell does not necessarily result in a productive infection. Productive infections occur only in cells able to support a complete viral multiplication cycle. Such cells are said to be permissive. In terms of cell cultures, the spectrum of permissive cells makes up the host range of a virus. However, the host range may also define the animal species that support a productive infection.

The host range of viruses may be wide, but it may also be very limited. For example, the host range of the parvovirus H-1 comprises humans, monkey, hamster and rat as animal species, and most cell types (fibroblasts, keratinocytes, lymphocytes) of human, monkey, hamster or rat origin. On the other hand, the host range of B19, another parvovirus, is restricted to humans and human erythroid precursor cells.

Infections of susceptible, but nonpermissive cells do not result in virus production. Three distinct types of such nonproductive infections have been described, and are referred to as abortive, latent and restrictive infections, respectively.

An abortive infection occurs in susceptible cells, which sustain some, but not all steps of the viral multiplication cycle. As stated earlier, after successful

entry of a virus into a cell, the viral genome has to become uncoated, amplified and expressed. Newly synthesized genomes have to be packaged into progeny particles that need to be released from infected cells.

Virus multiplication can be blocked at all stages of this cycle in susceptible, but nonpermissive cells. For example, the polyoma virus multiplication cycle is blocked at the stage of DNA replication and transcription in embryonal carcinoma cells lacking specific DNA-binding proteins. Similarly, the multiplication of the parvoviruses H-1 and MVM is blocked at the level of DNA and RNA synthesis in some cells that are refractory to virus propagation. Furthermore, some diploid cell strains infected with parvovirus H-1 are proficient in capsid assembly, but no DNA is packaged, and no particles are released from the cells. Cellular functions implicated in these last stages of infection have not yet been identified.

It should also be stated that infection of a permissive cell with a defective virus, lacking one or several essential viral functions, results in an abortive infection. During abortive infections, the virus may exert different cytopathic effects, depending on the viral functions that the host cells allow to be expressed. Hence, an abortive infection may result in cell death, if the virus was able to express its cytotoxic proteins, but may also be inapparent.

A latent infection consists of the persistence of viral genomes in infected cells for many cell generations. The viral genomes may persist in an integrated or in an episomal state or both. For example, retroviruses have been found as 'endogenous viruses' in human cells, and have been calculated to persist in these cells since approximately 40 million years. Such latent infections do not lead to host cell killing, but may nevertheless influence biologic characteristics of the cells. As an extreme example, persistence of tumor viruses is associated with cellular transformation leading to increased growth rates, abnormal cell morphology, altered cell metabolism and chromosomal aberrations.

Most viruses establishing latent infections can be rescued from their host cells, provided they have not become defective. Latent infections that can become productive are called persistent infections. To convert a latent into a productive infection, specific treatments have to be applied. For example, superinfection with adenovirus results in a productive infection of persistent adeno-associated viruses, and treatment with phorbol esters activates the multiplication cycle of persistent human immunodeficiency virus type 1 in the human macrophage cell line U1.

Finally, a rare type of nonproductive infection is the restrictive infection, that is observed in cell cultures where only a small subset of the cell population is permissive, or where the cells are only transiently permissive.

Virus Propagation in Whole Organisms

In initial studies in virology, the experimental tools for virus propagation and purification were whole laboratory animals or embryonated hen's eggs. Laboratory animals can be infected using the natural route of virus entry. To achieve this, the virus must be brought into contact with either the skin (e.g. papillomaviruses), the digestive tract (e.g. enteroviruses), the respiratory tract (e.g. orthomyxoviruses) or the conjunctiva (e.g. herpes simplex virus). Viral replication may be confined to the site of entry, or progeny virions may spread through the body (generally via the blood or lymphatic stream) with subsequent targeting to specific organs. Laboratory animals also can be infected by injecting the virus directly into specific organs (e.g. the brain for rabies virus). Several days or weeks after infection, the animals are killed, and cell-free extracts from the organs sustaining virus multiplication are used as a source of virus.

Furthermore, fertilized hen eggs can be infected after several days of incubation (depending on the stage of embryonic development required). Most viruses can be grown in the embryonic membranes of fertilized eggs, i.e. the yolk sac (e.g. herpesviruses), the chorion (e.g. poxviruses), the allantois (e.g. influenza virus) and the amnion (e.g. mumps virus). Although laboratory animals or embryonated eggs are still the most appropriate propagation systems for some viruses (animals for arboviruses, coxsackieviruses and rabies virus; eggs for orthomyxoviruses), they tend to be replaced by cell cultures, which are much more convenient to handle. For present-day virology, the use of animals is restricted mostly to research on viral pathogenesis and for production of vaccines.

Virus Propagation in Cell Culture

Multiplicity of infection, defective-interfering particles

The term 'cell culture' refers to *in vitro* cultures derived from dispersed cells taken from original tissues by enzymatical, mechanical or chemical disaggregation. Cultured cells may serve as hosts for the propagation of a number of viruses, provided these cells express all the factors allowing a complete viral replication cycle.

The first cells to be cultured were primary cells freshly isolated from animal tissues. The growth of

these cultures is restricted to five to ten cell generations at most, which limits their value for routine virus propagation. Such primary cultures contain a variety of cell types providing a broad viral spectrum. Thus, they represent a system in which as yet uncharacterized viruses can be propagated and isolated with a high probability of success, and are therefore valuable in virus diagnosis.

Cell lines derived from cancer tissues, capable of indefinite growth in culture, and strains of diploid cells prepared from human or animal tissues (in particular from embryos), capable of growing in culture up to 100 cell generations, contain cells of a specific type and allow the propagation of viruses under defined conditions. The choice of the most suitable cell line to propagate a virus will depend mainly on the host range and tissue specificity of the virus investigated. Continuous cell lines are useful for the propagation of viruses such as adenoviruses and rhinoviruses. Insect viruses (e.g. baculoviruses) can be propagated *in vitro* in immortal insect cells (e.g. SF cells, derived from *Spodoptera frugiperda* pupal ovarian tissue). Other viruses, like herpesviruses or enteroviruses, may preferentially be propagated in diploid cell strains of finite life.

However, attempts to propagate a series of viruses in cultured cells failed. This is mainly due to differences in cell behavior between cultured cells and their counterparts *in vivo*. Many of these differences stem from the dissociation of cells from a three-dimensional geometry and the lack of several systemic compounds involved in homeostatic regulation *in vivo*. As a consequence, cellular metabolism may not be truly representative of the tissue from which the cells were derived (see also below, 'Virus Propagation in Three-Dimensional Cell Culture Systems'). Hepadnaviruses, for example, require liver-specific receptor(s) to enter their host cells. In cell culture, only primary liver cells fulfill this requirement. Continuous hepatoma cell lines, however, do not express these receptor(s) in culture and therefore prohibit hepadnavirus propagation *in vitro*. Most data on the hepadnavirus replication cycle have therefore been obtained by genetic approaches using transfection of terminally redundant hepadnavirus DNA constructs, directing the synthesis of the RNA pregenome, into cultured hepatoma cells. This method allows only a single round of virus production, and precludes the study of early steps of the viral replication cycle such as the uncoating of the viral genome and its conversion into covalently closed circular DNA. Recently, receptor(s) required for internalization of hepadnavirus particles have been identified and cloned. The availability of cloned receptor genes now will allow cultured hepatoma cells to be genetically engineered in order to induce constitutive expression of these molecule(s) on their surface. Such cells subsequently may represent an efficient *in vitro* system for hepadnavirus propagation.

To obtain the best yield of progeny virus, it is important to determine the optimal conditions for the initial infection of the culture. One important parameter is the ratio of the number of cells to the number of infectious particles inoculated. This ratio is called multiplicity of infection (moi). A multiplicity of infection of two (moi = 2) defines an infection with an average of two infectious virus particles per exposed cell.

Most, if not all, viruses generate defective genomes, and, consequently, defective particles during their multiplication. This defectiveness is due to deletions in the viral genome, which render the virus incapable of expressing all the functions needed for a productive infection. These particles can arise because their defectiveness is complemented by genes of co-infecting 'wild-type' viruses. Therefore, the formation of defective particles is favored by infections at high moi. Adenovirus or parvovirus preparations may contain up to 1000 defective particles per infectious virion. In order to generate as few defective particles as possible, the standard protocol for the propagation of many viruses involves an initial infection at moi of 1×10^{-3} (i.e. one infectious virion per 1000 cells).

Infections are usually performed under cell culture conditions (37°C, pH 7.4, 5% CO_2) for 30 min to 1 h. The volume of the solution in which cells and viruses are brought together should be as small as possible in order to enhance the probability of host cell–virus contact. The presence of cations facilitates the adsorption process of most viruses. After infection, cell cultures are maintained at 37°C, except in the case of infections with viruses such as rhinoviruses or coronaviruses, which multiply best at 33°C, the temperature encountered in the nasal mucosa. After one or several multiplication cycles, progeny virus can be purified from the infected cultures.

Virus propagation in three-dimensional cell culture systems

As mentioned above for hepadnaviruses, papillomaviruses also cannot be propagated in conventional cell cultures. *In vivo,* papillomaviruses infect basal epithelial cells and establish their genomes as extrachromosomal elements. Following cell division, infected daughter cells migrate from the basal region and begin the process of differentiation. Upon terminal differentiation, papillomavirus genome amplification, late gene expression and virion assembly

are induced. Hence, attempts to propagate papillomaviruses in conventional cell cultures, which do not allow the cells to terminally differentiate, have not been successful, but were facilitated by the utilization of three-dimensional (3D) cell cultures (also called organotypic cultures or raft cultures). In organotypic cultures, cells of different lineages are recombined in a 3D matrix in spatial relationships and in experimentally determined ratios to recreate a component of the organ under study. Organotypic cultures of epithelial cells, for example, recreate important features, both morphological and physiological, of epithelial differentiation *in vitro* by raising the cells to an air–liquid interface, and even allow the achievement of terminal differentiation after treatment with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). When epithelial cells containing extrachromosomal papillomavirus genomes were grown in raft cultures, differentiation-dependent viral late gene expression, genome amplification and virion biosynthesis were observed.

Virus Purification

Viruses are purified from tissue (culture) homogenates, using various fractionation procedures. Different methods can be used depending on the physicochemical properties of the virions to be purified. Most virions are very sensitive to inactivation by heat, acid, alkali and lipid solvents. Accordingly, in most purification protocols, the virus is maintained at neutral pH and 4°C.

Generally, the supernatant of infected cell cultures provides a relatively clean virus suspension, but in some instances, viruses must be released by breaking up the cells by sonication, homogenization, or repeated freeze–thaw cycles. Thereafter, viruses can be concentrated and partially purified from cellular debris by adsorbing viral particles to erythrocytes, DEAE cellulose, aluminum hydroxide or ion-exchange resins. Subsequently, virions are eluted with buffers of specific pH and ionic strength. Viruses may also be precipitated with ammonium sulfate.

These partially purified viruses can be further separated from contaminants by physical methods, in particular by centrifugation. Differential centrifugation, which consists of multiple centrifugation cycles at increasing speed, is used to pellet first the contaminants and then the virions. Centrifugation through a cushion of a dense sucrose solution or through a preformed sucrose gradient (rate zonal centrifugation) separates viruses from contaminants based on their size and shape, i.e. their sedimentation coefficient. Equilibrium centrifugation in cesium chloride or potassium tartrate solutions is used to purify viruses as a function of their buoyant density.

Since virus purification aims at the elimination not only of cellular debris but also of defective viral material, equilibrium centrifugation may be the method of choice. This method allows the separation of infectious virions, defective particles, and empty capsids, which generally band at distinct densities (for example, infectious, defective and empty particles of the parvovirus H-1 have a density of 1.41, 1.38 and 1.32 g ml^{-1}, respectively). Before equilibrium centrifugation, it may be useful to treat the virus suspension with DNase, since contaminating cellular or viral DNA stick to some viruses and therefore would be copurified.

Virus preparations can be concentrated by ultracentrifugation, freeze-drying or dialysis against hydrophilic agents such as polyethylene glycol. Generally, concentration will be the last step of a purification protocol, but may be useful before centrifugation steps. Most viruses are heat labile. Therefore, virus preparations should be stored as cold as possible. As a rule, the half-life of many (enveloped) viruses will be years at −196°C, months at −70°C, days at 4°C and only hours at 20°C or minutes at 37°C.

Virus Titration

There is a variety of ways to determine the amount of virus in a preparation. Two types of methods should be distinguished: (1) infectivity assays that measure the amount of infectious virions in a preparation; and (2) particle assays that determine the number of all virus particles.

For lytic viruses, the most commonly used infectivity assay is the plaque assay. Monolayers of highly permissive cells are infected with serial dilutions of the virus suspension to be tested. After infection, the cells are overlaid with a semisolid agarose gel containing the culture medium, in order to restrict spread of progeny virus to cells next to the initially infected cells. Each infectious particle will give rise to a focus of infected cells, which can be seen as an area of CPE. The number of areas of CPE (plaques) within a cell monolayer can be easily counted after staining of the cell monolayer with a vital dye such as neutral red. Living cells stain red, and the areas of CPE appear as clear plaques against a red background (**Fig. 1**). From the number of plaques detected by this method, the number of infectious particles in a virus suspension can be calculated, and is expressed in terms of plaque-forming units (PFU) per milliliter.

To determine the titer of noncytopathogenic viruses, *in situ* hybridization assays can be used. Prerequisites for this test are the identification of permissive cells allowing the amplification of the viral

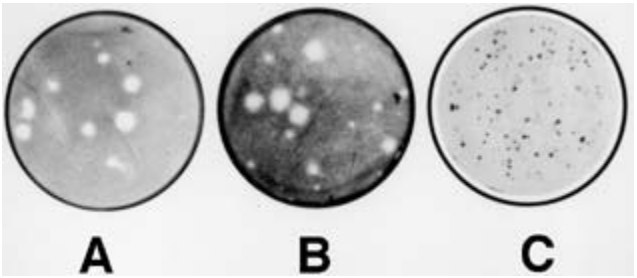


Figure 1 Virus titration. Plaque assay of the papovavirus simian virus type 40 (SV40) yieding clear-cut plaques (**A**) and of the parvovirus minute virus of mice (MVM) yielding more diffuse plaques (**B**). Areas of virus-induced cytopathic effect appear as clear plaques within the cell monolayer which has been stained with the vital dye Neutral Red. (**C**) An *in situ*-hybridization assay of an MVM-infected cell monolayer in which single cells containing amplified viral DNA appear as black dots. From the number of plaques (**A, B**) and dots (**C**), respectively, the total number of infectious particles in the virus suspension used for infection can be calculated. For more details see text.

DNA by factors of more than 200, and the availability of corresponding viral DNA that can be used as a probe. After infection of the cells, at the time when DNA amplification is maximal but before release of progeny virus, the monolayer is transferred to a nitrocellulose membrane. Cells attached to the membrane are subsequently lysed by alkali treatment, and the DNA (cellular and viral) is fixed to the membrane. A single cell that was infected and the viral DNA amplified, can be detected by autoradiography after hybridization of the filter-bound DNA with the corresponding radioactively labeled viral DNA (**Fig. 1**). Viral titers determined by this method give the number of viral particles within a virus suspension which can infect and replicate in a host cell, irrespective of whether this infection is productive or not.

Particle assays are much faster and simpler to handle. Two methods are widely used: the hemagglutination assay and particle counting by electron microscopy. Hemagglutination assays are based on the capacity of many viruses to adsorb to erythrocytes. Serial dilutions of a virus suspension are incubated with red blood cells. The viruses can bridge red blood cells, preventing them from precipitating. The virus dilution no longer capable of agglutinating erythrocytes gives a measure of the particle content of the suspension, and is expressed in terms of hemagglutinating units (HAU). This test is not very sensitive and reflects the presence of all the viral particles capable of binding to red blood cells, including defective particles and empty capsids.

Virus particles can also be counted directly by electron microscopy after negative staining. A known volume of virus suspension is deposited on a formvar-coated or carbon-coated copper grid, water and salts are removed, and viruses are subsequently negatively stained and counted. Since some virus may be lost during the washing and staining processes, it is convenient to add a marker to the virus suspension, e.g. in the form of a known concentration of latex particles. The number of latex particles counted enables the determination of particle loss during preparation and therefore allows the original virus particle concentration to be determined.

See also: **Cell structure and function in virus infections; Defective interfering viruses; Diagnostic techniques: Isolation and identification by culture and microscopy; Replication of viruses; Vectors: Animal viruses, Plant viruses; Viral receptors; Virus–host cell interactions.**

Further Reading

Freshney RI (1987) *Culture of Animal Cells. A Manual of Basic Technique*, 2nd edn. New York: Wiley–Liss.

Bacteria

Gail E Christie, Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia, USA

Introduction

Bacterial viruses, or bacteriophages, consist of a genome, either DNA or RNA, encapsidated in a protein coat. Occasionally this nucleoprotein capsid may also be surrounded by a membrane, derived from that of the host cell. The virus particle, or virion, serves to protect the nucleic acid and deliver it into a susceptible bacterial host. Expression of the viral genome results in infection of the host cell and production of new progeny phages. Bacteriophage multiplication depends upon the macromolecular synthetic machinery and energy generating systems of the infected bacterial cell. There are different outcomes of bacteriophage infection, depending on the nature of the interactions of a particular type of phage with the bacterial host. Virulent phages are those which invariably follow a lytic cycle of multiplication, resulting in the destruction of the infected cell and release of progeny phages. Temperate phages can undergo a similar lytic cycle, but also have an alternative mode of propagation in which their